RESEARCH NOTE

Last mated male sperm precedence in doubly mated females is not ubiquitous: evidence from sperm competition in laboratory populations of *Drosophila nasuta nasuta* and *Drosophila nasuta albomicans*

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Introduction

During reproduction, males can enhance their fitness by siring as many progeny as possible, while female fitness is more strongly affected by the quality of their offspring leading to the evolution of different reproductive strategies in males and females (Chapman 2006). Males transfer the sperm to female along with seminal mixture consisting of secretions from various tissues of their reproductive tract. These secretions are known to nourish sperm, help their storage in the female storage organs and protect them from other male's seminal fluid, thereby playing a crucial role in male's fitness (Avila *et al.* 2011). However, the female provides a competitive milieu for the sperm by mating with more than one male (Parker 1970; Gromko and Pyle 1978) and this is attributed to the presence of specialized sperm storage organs namely, a pair of spermathecae and the seminal vesicle. Thus, the female can store sperm from different males and utilize them to fertilize her eggs (Pitnick *et al.* 1999).

Sperm competition has been studied in wide range of taxa (Simmons and Fitzpatrick 2012). *Drosophila* has been extensively used as a model of choice to investigate the phenomenon of sperm competition (Gromko and Pyle 1978; Singh and Singh 2001; Avila *et al.* 2011). Availability of genetic markers and feasibility of its study in natural populations have enabled the proposal of various theories and models to explain the importance of this complex phenomenon (Clark *et al.* 1999). Most of the studies involving various species across taxa have shown that the sperm contributed by the latest male are more successful in fertilizing the eggs of a multiply mated female (Gromko and Pyle 1978; Singh and Singh 2001). Sperm competition poses selection pressure on males and contributes to divergence among closely related species (Simmons and Fitzpatrick 2012). Though sperm competition has been studied in different species of *Drosophila*, such a study in species having varying reproductive biology, cross-fertility and phylogenetic relatedness has not been made. *Drosophila nasuta* subgroup consisting of taxonomically closely related sibling species with varying degrees of reproductive isolation (cf. Ranganath and Aruna 2003) serves as an ideal model in this context. Though there are many species/subspecies in this subgroup, we have studied sperm competition by employing two closely related subspecies of the subgroup viz., *D. nasuta nasuta* and *D. n. albomicans*, due to availability of appropriate markers only in these two.

Materials and methods

Stocks

The wild type *D. n. nasuta* (stock no. 201.001; Coorg, India), *D. n. albomicans* (stock no. 202.001; Okinawa, Japan), and their spontaneous eye colour mutants *brown* obtained from Drosophila Stock Centre, Department of Studies in Zoology, University of Mysore, Mysore, India, were employed for the present experiments. These experimental stocks were built up and maintained on standard wheat cream agar medium in a vivarium at 22 ± 1°C, relative humidity of 70–80%, under uniform conditions of population density and resource availability in order to rule out the influence of these environmental factors on sperm competition. Virgin females and unmated males isolated every 3 h from these cultures were

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Table 1. Crosses conducted for sperm competition assay.

Cross	Female	P_1 male	P_2 male
1	*D. n. nasuta bw/bw*	*D. n. nasuta bw/bw*	*D. n. nasuta* bw^+/bw^+
2	*D. n. nasuta bw/bw*	*D. n. nasuta* bw^+/bw^+	*D. n. nasuta bw/bw*
3	*D. n. albomicans bw/bw*	*D. n. albomicans bw/bw*	*D. n. albomicans* bw^+/bw^+
4	*D. n. albomicans bw/bw*	*D. n. albomicans* bw^+/bw^+	*D. n. albomicans bw/bw*

maintained in fresh culture vials for seven days before using them for experiments.

Sperm competition assay

The first mating was conducted using a seven days old virgin *brown* (*bw/bw*) female and a seven days old wild-type male (bw^+/bw^+) by confining the pair in a fresh culture vial. After completion of mating, the male was discarded and female was allowed to lay the eggs. These vials with once mated females were labelled as vial 1. Only those females that laid fertilized eggs (confirmed by the presence of larvae in the vial) were selected for second mating.

The females from vial 1 were transferred to a fresh vial (vial 2) before the progeny started emerging. Since *D. n. nasuta* females were nonreceptive for remating until 17 days after mating once (Shruthi *et al.* 2012), the mated females were allowed to remate on 17th day with an unmated seven days old male (second male) in a fresh vial (vial 3). After remating, the males were discarded and females were allowed to lay eggs for 10 days with a change after five days (vial 4) in order to prevent mating of the progeny with female parent. The females producing only single type of progeny were discarded as it indicates lack of remating by that female. Similar procedure was followed for *D. n. albomicans* except that its females were allowed to remate on sixth day after first mating as most of them remate on sixth day after first mating (Shruthi *et al.* 2012). Different crosses set up in the present experiments are listed in table 1. The sperm competition was assessed as per Mueller *et al.* (2008) by scoring the progeny sired by two different males mated to the same female. The progenies of first and second males were designated as P_1 and P_2 respectively. The progeny of doubly mated females (from vials 3 and 4) were scored to assess the sperm competition and the standard formula P_1 = progeny of first male / total progeny and P_2 = progeny of second male / total progeny was applied to calculate the proportion of progeny sired by two different males (Boorman and Parker 1976; Singh and Singh 2001).

Statistics

Paired-sample *t*-test was carried out to assess difference between first and second male progenies in all the crosses independently. The difference in the pattern of sperm competition among different crosses was assessed by two-way analysis of variance (ANOVA); where the crosses were considered as factor 1 and the proportion of progeny was considered as factor 2 and the interaction between these two factors was also considered. All the statistical tests were carried out using SPSS software (ver. 16.0).

Results

The proportion of first and second male progenies calculated for all the crosses have been compiled in table 2. Paired-sample *t*-test conducted to check for the differences between progeny of first and second male for each cross independently revealed that P_1 was significantly higher than P_2 in all four crosses. In cross 1, *brown D. n. nasuta* female was first mated with *brown* male followed by mating with wild-type male. In cross 2, *brown D. n. nasuta* female was allowed to mate with wild-type male first; followed by *brown* male. In both these crosses, irrespective of the order of male parent, the P_1 was always significantly higher than P_2. Similar situation was observed even in crosses involving *D. n. albomicans*.

Two-way ANOVA was conducted to assess the differences in the proportion of progeny of first and second male

Table 2. Proportions of first male and second male progeny of doubly mated female and the results of paired-sample *t*-test carried out independently for each cross.

Cross	Number of doubly mated females scored	Paired-sample *t*-test				
		P_1	P_2	*t*	df	*P*
1	58	0.62 ± 0.03	0.38 ± 0.03	3.498	57	0.001
2	31	0.69 ± 0.03	0.31 ± 0.03	5.988	30	0.0001
3	20	0.70 ± 0.07	0.30 ± 0.07	2.873	19	0.010
4	41	0.70 ± 0.05	0.29 ± 0.05	4.563	40	0.0001

P value significant at 0.05% level.

Table 3. Two-way ANOVA carried out for assessing interaction between crosses and proportions of first and second male progeny.

	df	Mean square	*F*	*P*
Crosses	3	0.0005	0.001	1.000
Proportion of progeny	1	8.296	120.04	0.0001
Crosses × proportion of progeny	3	0.157	2.275	0.08

P significant at 0.05% level.

considering all the crosses simultaneously. The interaction between crosses and proportion of progeny was not statistically significant. This showed that both *D. n. nasuta* and *D. n. albomicans* exhibit similar pattern of sperm precedence (table 3).

Discussion

We have found that in both the *D. nasuta* subgroup members, the first male to mate showed precedence in fertilizing most of the eggs in a doubly mated female, irrespective of the order of wild type and *brown* males used for the cross. This preferential utilization of first male sperm in presence of latest male's sperm is in contrast to sperm competition observed in other species. Similar studies in other species of *Drosophila* have revealed that the second male to mate fertilizes most of the eggs of a doubly mated female (Parker 1970; Gromko and Pyle 1978; Singh and Singh 2001). Several hypotheses suggest that the last male seminal fluid displaces the stored sperm from the female reproductive tract and ensure fertilization success of its own sperm (Avila *et al.* 2011). Further, Manier *et al.* (2010) have shown the live fluorescently tagged sperm displacing stored sperm from the sperm storage organs.

The subspecies used for the present investigations are cross-fertile under laboratory conditions; though in nature they are allopatric. In spite of their cross-fertility, they differ with respect to various components of their reproductive biology (cf. Ranganath and Aruna 2003). We have found that *D. n. nasuta* females have prolonged remating latency of 17 days when compared with *D. n. albomicans* which remates just in six days (Shruthi *et al.* 2012). Hence, in the present study difference in remating latency was considered while allowing the second male to pair with once mated female. From the present study it is evident that though *D. n. nasuta* and *D. n. albomicans* vary in their remating behaviour, they show similar pattern of sperm competition.

Quantity of male seminal content influences the outcome of sperm competition and male accessory gland secretions form the major bulk of *Drosophila* seminal content (Avila *et al.* 2011). In the present experiments seven days old flies were employed, since *D. nasuta* subgroup males accumulate maximum quantity of accessory gland proteins seven days after eclosion (Ravi Ram and Ramesh 1999). Further, we employed seven days' old unmated males for both first mating and remating to rule out the possible variation due to male's age. In certain studies, the progeny of first male before remating have also been considered to assess sperm competition (Clark *et al.* 1999; Mueller *et al.* 2008); in the present study only the progeny of doubly mated females were scored as per Singh and Singh (2001) to score the progeny produced as a consequence of competition between sperm from different males. Though *D. n. nasuta* and *D. n. albomicans* females differ in the time required to regain their receptivity for remating drastically, they show preferential use of first male sperm after remating. Mack *et al.* (2003) have proposed that as the female's age increases, her life expectancy comes down and she tends to use sperm from her previous mate rather than using fresh sperm from her last mate. In the present study, we have documented that in *D. n. nasuta*, the remating latency is quite prolonged, obviously by the time it becomes receptive for remating, the female's age increases correspondingly. We can presume that due to increase in female's ageing, it would use the sperm which are already present in her storage organs rather than selectively using fresh sperm from her remating. Thus first male precedence in *D. n. nasuta* corresponds with the proposal of Mack *et al.* (2003). However, same explanation cannot be extended to *D. n. albomicans* which also shows first male precedence; since, in this case the remating latency is only six days after first mating which is almost similar to that of *D. melanogaster* (seven days) (Gromko and Pyle 1978) and few other species of *Drosophila* such as *D. ananassae* (seven days after first mating), *D. littoralis* and *D. montana* (six days after first mating) which exhibit second male precedence (Aspi 1992; Singh and Singh 2001).

The *brown* mutation in *D. n. nasuta* and *D. n. albomicans* is not isolocus and therefore reciprocal crosses conducted between *brown* of *D. n. nasuta* and *brown* of *D. n. albomicans* yields only wild-type phenotype (Ashadevi *et al.* 2005). Hence, the competition between conspecific and heterospecific sperm and the pattern of sperm precedence in the F_1 hybrids could not be included for the present study and thus we focussed only on intraspecific sperm competition involving these two subspecies. Further, using glue protein as marker, Ramesh and Shivanna (2001), have documented that 87.5% of *D. n. nasuta* and 90% of *D. s. neonasuta* females from natural populations have sperm from more than one male. Thus, like any other species of *Drosophila*, *D. n. nasuta* and *D. n. albomicans* also exhibit female remating in the laboratory populations. However, they differ from each other in their female remating latency and differ from the other species in having first male precedence.

At the moment, though we are not aware of what determines or regulates the first male precedence in *D. n. nasuta* and *D. n. albomicans*, further experiments in other members and in hybrids of cross compatible members of *nasuta* subgroup may provide valuable information in this regard. However, such studies demand genetic markers which have to be generated in due course.

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